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Abstract

Arsenic is commonly present in the natural environment and is also used as a feed additive for animal production. Poultry is a major reservoir for *Campylobacter jejuni*, a major food-borne human pathogen causing gastroenteritis. It has been shown that *Campylobacter* isolates from poultry are highly resistant to arsenic compounds, but the molecular mechanisms responsible for the resistance have not been determined, and it is unclear if the acquired arsenic resistance affects the susceptibility of *Campylobacter* spp. to other antimicrobials. In this study, we identified a four-gene operon that contributes to arsenic resistance in *Campylobacter*. This operon encodes a putative membrane permease (ArsP), a transcriptional repressor (ArsR), an arsenate reductase (ArsC), and an efflux protein (Acr3). PCR analysis of various clinical *C. jejuni* isolates indicated a significant association of this operon with elevated resistance to arsenite and arsenate. Gene-specific mutagenesis confirmed the role of the *ars* operon in conferring arsenic resistance. It was further shown that this operon is subject to regulation by ArsR, which directly binds to the *ars* promoter and inhibits the transcription of the operon. Arsenite inhibits the binding of ArsR to the *ars* promoter DNA and induces the expression of the *ars* genes. Mutation of the *ars* genes did not affect the susceptibility of *C. jejuni* to commonly used antibiotics. These results identify the *ars* operon as an important mechanism for arsenic resistance and sensing in *Campylobacter*.

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Identification of an Arsenic Resistance and Arsenic-Sensing System in *Campylobacter jejuni*▽†

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Arsenic is commonly present in the natural environment and is also used as a feed additive for animal production. Poultry is a major reservoir for *Campylobacter jejuni*, a major food-borne human pathogen causing gastroenteritis. It has been shown that *Campylobacter* isolates from poultry are highly resistant to arsenic compounds, but the molecular mechanisms responsible for the resistance have not been determined, and it is unclear if the acquired arsenic resistance affects the susceptibility of *Campylobacter* spp. to other antimicrobials. In this study, we identified a four-gene operon that contributes to arsenic resistance in *Campylobacter*. This operon encodes a putative membrane permease (ArsP), a transcriptional repressor (ArsR), an arsenate reductase (ArsC), and an efflux protein (Acr3). PCR analysis of various clinical *C. jejuni* isolates indicated a significant association of this operon with elevated resistance to arsenite and arsenate. Gene-specific mutagenesis confirmed the role of the *ars* operon in conferring arsenic resistance. It was further shown that this operon is subject to regulation by ArsR, which directly binds to the *ars* promoter and inhibits the transcription of the operon. Arsenite inhibits the binding of ArsR to the *ars* promoter DNA and induces the expression of the *ars* genes. Mutation of the *ars* genes did not affect the susceptibility of *C. jejuni* to commonly used antibiotics. These results identify the *ars* operon as an important mechanism for arsenic resistance and sensing in *Campylobacter*.

Arsenic is a toxic metalloid present in the natural environment. At high levels, arsenic is toxic to most cells, including microbial organisms (32, 33). To survive this toxicity, bacterial organisms have evolved multiple mechanisms for arsenic detoxification, including extrusion mediated by efflux transporters, reduction of arsenate [As(V)] to arsenite [As(III)], which is subsequently extruded by efflux transporters, and methylation of As(III) by *S*-adenosylmethionine methyltransferase (39, 41). These resistance mechanisms are encoded by various *ars* genes, and the best-characterized ones include *arsR*, *arsA*, *arsB*, *arsC*, *arsD*, *arsH*, and *arsM*. The *arsR* gene encodes an As(III)-responsive transcriptional repressor, which controls the expression of other *ars* genes by binding to target promoters (42, 55). ArsA and ArsB form an oxyanion-translocating complex, in which ArsA functions as an ATPase, while ArsB is a membrane efflux transporter (41). In addition, ArsB can function alone (without ArsA) in the extrusion of arsenite (9). *arsC* encodes a arsenate reductase, which converts arsenate to arsenite (20). ArsD was previously thought to be an inducer-independent regulatory protein, but recent work indicated that it functions as an arsenic metallochaperone transferring As(III) to ArsA and increasing the rate of arsenic extrusion (26, 27, 57). *arsH* encodes an NADPH-flavin mononucleotide oxidoreductase and has been shown to be involved in arsenic resistance in *Yersinia enterocolitica* and *Sinorhizobium meliloti* (31, 59, 60). The newly identified arsenic resistance gene *arsM* encodes an arsenite *S*-adenosylmethionine methyltransferase that methylates arsenite to volatile trimethylarsine, contributing to arsenic detoxification in bacteria (39). In bacteria, the *ars* genes can be carried on either plasmids or chromosomes and are often organized in operons, such as *arsRBC*, *arsRABC*, and *arsRDABC*, but in some cases the *ars* genes exist singly (3–5, 10, 29, 45, 50).

Campylobacter jejuni is a leading cause of bacterial gastroenteritis in humans and is estimated to be responsible for 400 to 500 million cases of diarrhea each year worldwide (43). As a food-borne pathogen, *C. jejuni* is widely distributed in food-producing animals, including both livestock and poultry (18). In animal reservoirs, *C. jejuni* colonizes the intestinal tracts of animals and usually does not cause clinical diseases. But once transmitted to a human host, it can cause severe gastroenteritis (30). Transmission of *Campylobacter* bacteria from animals to humans is mainly via contaminated poultry, water, and raw milk (18). Among domestic animals, poultry is considered the major reservoir for *C. jejuni* (61). The high prevalence of *C. jejuni* on chicken farms suggests that this organism is well adapted in the poultry production system, but the mechanisms involved in the adaptation are poorly understood. Understanding the adaptive mechanisms may facilitate the development of intervention strategies to control the prevalence of this pathogenic organism.

During the past decade, *Campylobacter* species have become increasingly resistant to clinically important antibiotics such as fluoroquinolones and macrolides (13, 35). Antimicrobials including organoarsenical compounds, such as 3-nitro-4-hydroxyphenylarsonate (roxarsone), are commonly used in poultry production in the United States and other countries

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(6). Roxarsone is given to broiler chickens as a feed additive to promote growth and control coccidiosis, an intestinal disease caused by *Eimeria* parasites (6). The roxarsone fed to chickens is excreted into feces and subsequently converted to inorganic arsenic (arsenite and arsenate) via the bioconversion processes in poultry litter (8, 49). Since *C. jejuni* colonizes the intestinal tracts of poultry, it is likely that *Campylobacter* bacteria are often exposed to arsenic compounds and have adapted to the selection pressure. Indeed, a recent study by Sapkota et al. indicated that *Campylobacter* isolates from conventional poultry products were significantly more resistant to roxarsone than those isolates from antimicrobial-free poultry products (44). Despite these observations, the molecular mechanisms involved in arsenic resistance in *Campylobacter* are unknown, and the impact of the acquired arsenic resistance on the susceptibility of *Campylobacter* spp. to other antimicrobials, including clinically important antibiotics, has not been determined.

Previously, Ahmed et al. compared the gene contents of *C. jejuni* 81116 and NCTC 11168 using subtractive hybridization and reported the presence of *arsB* and *arsC* in 81116 but not in NCTC 11168 (2). Recently, the genomic sequences of several *C. jejuni* strains were determined, which revealed the presence of *ars* gene homologs located on *Campylobacter* chromosomes (12, 17, 34, 36, 38). Comparison of the genomic sequences of strains NCTC 11168, 81-176, and RM1221 revealed that a four-gene operon (CJE1730, CJE1731, CJE1732, and CJE1733) is present in RM1221, and three of the open reading frames (ORFs) share significant sequence homology with known *ars* genes, suggesting that this operon may be involved in arsenic resistance in *Campylobacter*. In this study, we determined the contribution of this operon to arsenic resistance, its induction by arsenic, and its regulation by the transcriptional regulator ArsR. In addition, we examined if the *ars* operon contributes to the resistance to nonarsenic antimicrobials in *Campylobacter*. Findings from this study identify a new mechanism involved in arsenic resistance and sensing in *Campylobacter* and provide new insights into the adaptation mechanisms of *Campylobacter* bacteria in animal reservoirs.

MATERIALS AND METHODS

Plasmids, bacterial strains, and growth conditions. In total, 27 *C. jejuni* isolates were used in this study. Of these strains, 10 (CT1-8, CT3-7, CT3-2, CT2-2, CT1-20, CT1-17, CT4-3,CT4-12, CT4-23, and CT4-22) were isolated from turkeys, 9 (CB2-1, CB2-2, CB3-6, CB4-4, CB8-16, CB7-22, CB6-27, CB5-28, and RM1221) from chickens, and 7 (NCTC 11168, 81-176, F12469, 214274, Clev9100, 79034, and H52022) from humans. ATCC 33560 was from American Type Culture Collection (Rockville, MD). These isolates were routinely grown in Mueller-Hinton (MH) broth (Difco) or on MH agar at 42°C under microaerophilic conditions (5% O_2, 10% CO_2, and 85% N_2). Plasmids, various mutant constructs, and cloning hosts used in this study are listed (see Table S1 in the supplemental material).

Chemical compounds and antibiotics. The chemicals and antibiotics used in this study included arsenite ($NaAsO_2$), arsenate (Na_2HAsO_4), roxarsone, ampicillin, erythromycin, streptomycin, tilmicosin, ciprofloxacin, kanamycin, oxytetracycline, ceftiofur, and polymyxin B. They were purchased from Sigma Chemical Co. (St. Louis, MO).

Antimicrobial susceptibility tests. The MICs of various arsenic compounds and antibiotics against *C. jejuni* strains were determined using the agar dilution susceptibility testing method as described by CLSI (7) and the broth microdilution method as described previously (25). Each MIC test was repeated at least three times.

PCR analysis. To determine the distribution of the arsenic resistance operon, *arsC*-specific primers (arsCF and arsCR) and *acr3*-specific primers (cje1733F and cje1733R) were designed from the genomic sequence of *C. jejuni* RM1221 (12) and used in PCR analyses with the genomic templates of different *C. jejuni* strains and Ex *Taq* polymerase (TaKaRa Bio Inc., Japan). PCR was performed in a volume of 50 μl containing 2 μM of primers, 250 μM of deoxynucleoside triphosphates, and 5 U of Ex *Taq* polymerase. Annealing temperatures and elongation times varied according to the primers used and the expected sizes of products.

Sequence determination of the *ars* operon in *C. jejuni* strain CB5-28. To facilitate the functional characterization of the *ars* operon, the *ars* genes in strain CB5-28 were amplified by PCR and then the PCR products were subsequently sequenced. The primers used in the PCR were designed from the published genomic sequence of RM1221 (12). The entire *ars* operon and its flanking region was first PCR amplified with primer pair Ars1_F and Ars3_R (see Table S2 in the supplemental material) using the *Pfu* DNA polymerase, and the product was subsequently sequenced using primers Ars1_F, Ars1_R, Ars2_F, Ars2_R, Ars3_F, and Ars3_R (see Table S2 in the supplemental material).

Construction of the *arsR*, *arsC*, and *acr3* mutants. In order to evaluate the function of the operon in arsenic resistance, isogenic *arsR*, *arsC*, and *acr3* mutants were constructed. Initially, we tried to use *C. jejuni* RM1221, whose genomic sequence was known (12), in order to construct the mutants, but RM1221 was not transformable by natural transformation or electroporation (data not shown), prohibiting the generation of the desired mutants. Thus, *C. jejuni* CB5-28, which harbors an *ars* operon identical to that of RM1221 (Fig. 1), was used for mutagenesis in this study. The plasmids and primers used in this work are listed (see Tables S1 and S2 in the supplemental material). To construct an isogenic *arsC* mutant of strain CB5-28, primers arsCF and arsCR were used in PCR analysis (with the *Pfu* DNA polymerase) to amplify a 1.7-kb fragment that spans from 800 bp upstream of the start codon of *arsC* to 500 bp downstream of the stop codon of *arsC*. Within *arsC*, there is a unique EcoRV restriction site in the middle of the ORF. The PCR product was cloned into the SmaI site of pUC19, resulting in the construct pARSC, which was subsequently digested by EcoRV. Primers kanNCOF and kanNCOR (see Table S2 in the supplemental material) were used to amplify the 1.0-kb kanamycin resistance cassette (Kan^r) from plasmid pMW10 (54). The Kan^r cassette was ligated to EcoRV-digested pARSC, yielding the construct pARSCK, in which the Kan^r cassette was inserted into the *arsC* in the same direction of transcription. The pARSCK plasmid, as a suicide vector, was electroporated into *C. jejuni* CB5-28. Transformants were selected on MH agar containing 50 μg/ml of kanamycin. Insertional mutagenesis of the *arsC* gene in the *C. jejuni* transformants was confirmed by PCR. The *arsC* mutant of CB5-28 was named LP001 in this study (see Table S1 in the supplemental material). To create the isogenic *arsC* mutant in strains CB3-6 and F12469, the insertional mutation in *arsC* of CB5-28 was transferred into strains CB3-6 and F12469 by natural transformation as described previously (53).

An isogenic *acr3* mutant of strain CB5-28 was also constructed by insertional mutagenesis. Primers cje1733F and cje1733R were used to amplify the entire *acr3* gene, which contains a unique EcoRI restriction site in the middle of the ORF. The PCR product was cloned into pGEM-T (Promega, Madison, WI), resulting in pCJE1733, which was further digested with EcoRI and treated with the Klenow fragment (TaKaRa). The 1.0-kb chloramphenicol resistance cassette (*cat*) was amplified with primers catF and catR (see Table S2 in the supplemental material) from plasmid pUOA18 (52) and ligated to EcoRI-digested pCJE1733, generating pCJE1733C, which was then electroporated into *C. jejuni* CB5-28. Transformants were selected on MH agar plates containing 4 μg/ml of chloramphenicol. The insertion of the *cat* gene into *acr3* was confirmed by PCR using primers cje1733F and cje1733R. The *acr3* mutant of CB5-28 was named LP002 in this study (see Table S1 in the supplemental material).

To generate a nonpolar mutation in *arsR*, the in-frame deletion method described by Hendrixson et al. (16) was used in this study. The pDRH173 and pDRH265 plasmids that carry *rpsL*(Sm), which confers resistance to streptomycin, and a *cat*::*rpsL* cassette, respectively (16), were used for the in-frame deletion. pDRH173 was electroporated into CB5-28 to replace the endogenous *rpsL* with *rpsL*(Sm) to generate a streptomycin-resistant CB5-28 strain. The *arsR* gene was cloned into pGEM-T (Promega). The *cat*::*rpsL* cassette was prepared by digesting pDRH265 with SmaI and subsequent purification with agarose gel extraction (QIAquick gel extraction kit; Qiagen, Valencia, CA). The cassette was then cloned into the XbaI site of *arsR* in pGEM-T to yield the pArsRC plasmid. pArsRC was electroporated into the streptomycin-resistant CB5-28 strain, and transformants were selected on MH agar containing chloramphenicol (10 μg/ml). The transformants were then transferred to MH agar plates containing streptomycin to screen for streptomycin-susceptible transformants. A strain resistant to chloramphenicol, but sensitive to streptomycin, was obtained and

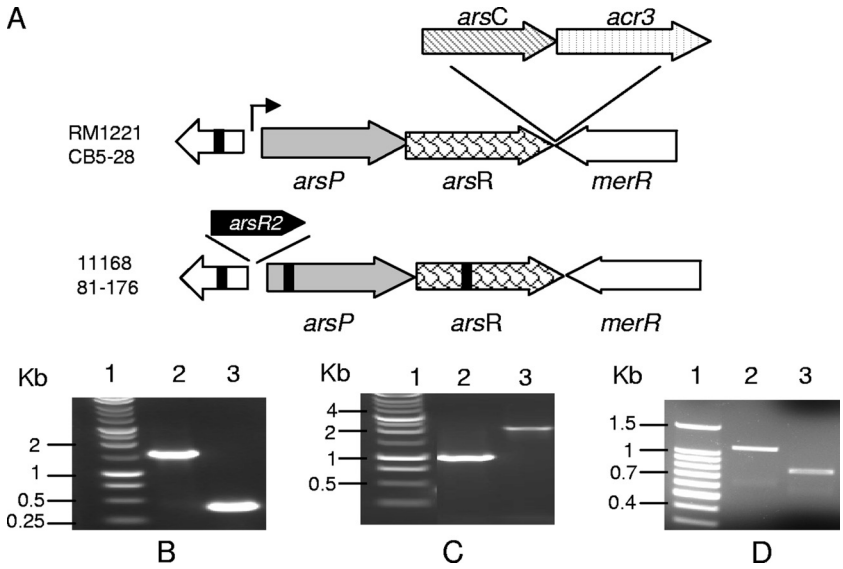

FIG. 1. Genomic organization of the *ars* operon and confirmation of the generated *ars* mutants. (A) Diagram of the *ars* genes in *C. jejuni* strains RM1221, CB5-28, NCTC 11168, and 81-176. ORFs are indicated by boxed arrows. The single promoter in front of *arsP* is depicted by a curved arrow. In strains NCTC 11168 and 81-176, *arsC* and *acr3* are absent and *arsP* and *arsR* are degenerate due to frameshift mutations (indicated by black strips in the genes). An additional *arsR* homologue in NCTC 11168 and 81-176 (named *arsR2*) is indicated with a black arrow. (B) PCR confirmation of the *arsC*::Kan^r insertion in LP001. Lane 1, 1-kb DNA ladder; lane 2, LP001; and lane 3, wild-type CB5-28. (C) PCR confirmation of the *acr3*::*cat* insertion in LP002. Lane 1, 1-kb DNA ladder; lane 2, wild-type CB5-28; and lane 3, LP002. (D) PCR confirmation of the in-frame deletion in *arsR* in LP004. Lane 1, 100-bp DNA ladder; lane 2, wild-type CB5-28; and lane 3, LP004.

named LP003. The sensitivity of LP003 to streptomycin was due to the recessive nature of *rpsL*(Sm) in the presence of a wild-type *rpsL* gene. To generate a deletion in *arsR*, a 576-bp fragment upstream of *arsR* and an 804-bp fragment downstream of *arsR* were PCR amplified with primer pairs DelF1/DelR1 and DelF2/DelR2, respectively. The DelR1 and DelF2 primers were designed to contain a 21-nucleotide overlapping sequence to be used as an annealing region in the subsequent PCR ligation (see Table S2 in the supplemental material). The PCR ligation linked the two fragments into one product, in which an in-frame deletion (from nucleotide 4 to 297) within *arsR* was generated. The product of PCR ligation was cloned into pGEM-T, and the plasmid construct was electroporated into the LP003 strain. Transformants were initially selected on MH agar containing streptomycin (2 mg/ml) and then screened for chloramphenicol sensitivity by being grown on MH agar containing chloramphenicol (10 μg/ml). In-frame deletion of *arsR* was confirmed by PCR and sequencing. The *arsR* in-frame deletion mutant of CB5-28 was named LP004 (see Table S1 in the supplemental material).

RT-PCR and real-time qRT-PCR. To determine if the *ars* operon is inducible by arsenic, *C. jejuni* CB5-28 was cultured in MH broth with or without added arsenite and arsenate for 16 h. The final concentrations of arsenite and arsenate in the culture were 0.25 times their corresponding MIC in CB5-28. Total RNA was extracted from four replicate cultures from each experiment and the culture experiment repeated three times. To determine if the deletion of *arsR* affected the expression of *arsP*, *arsC*, and *acr3*, wild-type CB5-28 and LP004 (Δ*arsR*) were grown in MH broth under identical conditions overnight to the late log phase. Total RNA was extracted from four replicate cultures in each experiment, which was repeated three times. RNA was extracted using the RNeasy mini kit (Qiagen) according to the procedure supplied with the product. The RNA preparations were further treated with the Turbo DNA-free kit (Ambion) to eliminate DNA contamination in each preparation. The lack of DNA in each RNA sample was confirmed by PCR. For real-time quantitative reverse transcription-PCR (qRT-PCR), primer pairs 1730F1/1730R1, 1732F1/1732R1, and 1733F1/1733R1 (see Table S2 in the supplemental material) specific for *arsP*, *arsC*, and *acr3*, respectively, were designed using the Primer3 online interface (http://frodo.wi.mit.edu/). Each amplicon was analyzed by the Mfold server (http://mfold.bioinfo.rpi.edu/) to avoid secondary RNA structures and hairpin loops. Real-time RT-PCR analyses were conducted using the iScript one-step RT-PCR kit with SYBR green (Bio-Rad) along with the MyiQ iCycler real-time PCR detection system (Bio-Rad, Hercules, CA) as described in a previous publication (24). The 16S rRNA gene was used for the normalization. To confirm whether inactivation of *arsC* has a polar effect on downstream gene *acr3*, RT-PCR was performed with Superscript II reverse transcriptase (Gibco BRL) according to the manufacturer's instructions.

Cloning, expression, and purification of rArsR. Primers ArsRF1 (containing a BamHI site) and ArsRF2 (containing an XmaI site) were used to amplify the full-length *arsR* from CB5-28. The product was cloned into BamHI- and XmaI-digested pQE30 expression vector (Qiagen). The construct was transformed into *Escherichia coli* JM109. The plasmid in the *E. coli* clone expressing recombinant His_6-ArsR (rArsR) was sequenced, which confirmed the correct fusion of ArsR to the His_6 tag and the lack of mutations in the cloned *arsR*. Induction and purification of the His-tagged rArsR under native conditions were performed according to the instructions provided with the pQE30 expression vector. Once eluted from the Ni^{2+}-nitrilotriacetic acid column, the rArsR was immediately passed through a PD-10 desalting column (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) and stored at −80°C in single-use aliquots. The concentration of the purified rArsR was determined using the bicinchoninic acid protein assay kit (Pierce, Rockford, IL), with bovine serum albumin as a standard.

EMSA. To determine the binding of ArsR to the promoter region of the arsenic resistance operon, an electrophoretic mobility shift assay (EMSA) was performed using the method described in a previous publication (19). The 164-bp promoter region of the *ars* operon was amplified using primers gelF and gelR (see Table S2 in the supplemental material) from *C. jejuni* CB5-28. The DNA fragment was then labeled at the 3′ end with digoxigenin (DIG)-11-ddUTP using the DIG oligonucleotide 3′-end-labeling kit (Roche Molecular Biochemicals, Basel, Switzerland). A 190-bp intragenic DNA fragment of *arsP* was amplified with primers NCF and NCR (see Table S2 in the supplemental material), labeled with DIG-11-ddUTP, and used as a negative control DNA for the EMSA. For the competition experiments, a 50-bp DNA fragment containing the inverted repeat sequence in the *ars* promoter was generated by annealing complementary oligonucleotides CompF and CompR (see Table S2 in the supplemental material). The two oligonucleotides were dissolved in STE buffer (10 mM Tris, pH 8.0, 50 mM NaCl, 1 mM EDTA) and were mixed in an equal molar ratio. The mixture was heated to 94°C and slowly cooled. This double-stranded DNA was used as competitor in the EMSA. Also, the competition assay was carried out with the negative-control DNA amplified by primer NCF and NCR (described above). To determine if arsenite and arsenate inhibited the binding of ArsR to the promoter DNA, various amounts of arsenic (0.01, 0.1, and 1 mM for arsenite; and 1, 8, and 32 mM for arsenate) were added to the binding reaction mixtures.

Primer extension analysis. To identify the transcriptional start site of the *ars* operon, oligonucleotide PEA-R (see Table S2 in the supplemental material),

TABLE 1. MICs (μg/ml) of arsenic compounds against different *C. jejuni* strains and the presence of *arsC* and *acr3* in the strains as determined by PCR[a]

Strain	MIC (μg/ml)			Presence of *arsC* and *acr3*
	Rox	As(III)	As(V)	
RM1221	128	64	2,048	+
CT1-8	128	64	2,048	+
CT3-7	16	64	128	+
CT2-2	128	16	512	+
CT1-20	32	64	128	+
CT4-3	128	64	256	+
CT4-12	64	8	512	+
CT4-23	64	8	512	+
CB8-16	64	32	1,024	+
CB7-22	16	32	512	+
CB6-27	16	32	1,024	+
CB5-28	64	64	1,024	+
CB3-6	16	64	512	+
79034	128	16	1,024	+
F12469	128	64	2,048	+
214274	32	32	512	+
H52022	256	8	256	+
NCTC 11168	8	4	128	−
81-176	8	8	128	−
ATCC 33560	8	8	64	−
CT3-2	64	16	32	−
CT1-17	64	16	128	−
CT4-22	32	16	128	−
CB2-1	16	8	64	−
CB2-2	32	8	64	−
CB4-4	256	16	32	−
Clev9100	128	8	256	−

[a] PCR analysis was performed using *arsC*- and *acr3*-specific primers. Rox, roxarsone; +, positive PCR for both *arsC* and *acr3*; −, negative PCR for both *arsC* and *acr3*.

complementary to the region immediately downstream of the start codon of the *arsP* gene, was synthesized and 5′-end labeled with 5-6-carboxyfluorescein by IDT (Coralville, IA). Total RNA was isolated from a log-phase culture of the *arsR* mutant (LP004) of CB5-28. Use of the mutant strain for primer extension was based on the expectation that the mutant strain would produce more mRNA transcripts of the *ars* operon than the wild-type strain due to the deletion of *arsR*, thus yielding a better signal in the assay. The procedures for the primer extension reaction and subsequent detection of the cDNA product were described in previous publications (15, 19).

Statistical analysis. The MIC data for arsenite, arsenate, and roxarsone in various *C. jejuni* strains were analyzed according to the PCR result of the *ars* operon. Statistical comparison of the MICs of the strains with the *ars* operon and those without the operon was performed by using the two-sample Wilcoxon-Mann-Whitney test using the SAS software. A P value of <0.05 is considered significant.

Nucleotide sequence accession number. The sequences for the *ars* genes have been deposited in GenBank under accession no. EU916833.

RESULTS

Identification of the *ars* operon in *Campylobacter*. As the first step to identify the genetic basis responsible for arsenic resistance in *C. jejuni*, we compared the MICs of arsenic compounds in three sequenced strains, including NCTC 11168 (34), 81-176 (17), and RM1221 (12). As shown in Table 1, the MICs of roxarsone, arsenite, and arsenate in strains NCTC 11168 and 81-176 were 8, 4 to 8, and 128 μg/ml, respectively, while their MICs in RM1221 were 128, 64, and 2,048 μg/ml, respectively. This finding indicated that strain RM1221 had a significantly higher level of resistance to arsenic compounds than the other two strains. Comparison of the genomic sequences of RM1221, NCTC 11168, and 81-176 revealed a locus potentially involved in the elevated resistance to arsenic compounds (Fig. 1A). In RM1221, there is a four-gene cluster including CJE1730, CJE1731, CJE1732, and CJE1733. Within the locus, CJE1730 (315 amino acids [aa]) encodes a putative membrane permease of an unknown function. We designate CJE1730 and its orthologs in other *C. jejuni* strains as *arsP* (arsenic resistance permease) in this study. CJE1731 (105 aa) is separated from CJE1730 by 10 bp and was annotated as *arsR* due to its high sequence homology with the transcriptional regulators of the ArsR family (12). The third gene CJE1732 (423 aa) encodes a putative cytoplasmic arsenate reductase and was annotated as *arsC* (12). The start codon of *arsC* overlaps with the stop codon of *arsR*. CJE1733 (347 aa) overlaps with *arsC* by six nucleotides, encodes a putative arsenic resistance membrane transporter of the Acr3 family (12), and is designated as *acr3* in this study (Fig. 1A). The *acr3* gene shares little sequence homology with known *arsB* genes. Based on their sequence homologies with genes encoding proteins of known functions and the fact that the four genes are clustered together on the chromosome (Fig. 1A), we designated this four-gene locus as the *ars* operon in *C. jejuni*. RT-PCR using primers spanning the junctions of the adjacent genes in the operon confirmed cotranscription of the *ars* genes (data not shown). Compared with RM1221, NCTC 11168 and 81-176 do not have *arsC* and *acr3* in the *ars* locus (Fig. 1A). The *arsR* gene in NCTC 11168 and 81-176 is degenerated and split into two small ORFs due to a frameshift mutation caused by a single-nucleotide deletion in the coding sequence of *arsR*. In addition, the *arsP* gene in strains NCTC 11168 and 81-176 is also degenerated as result of a single-nucleotide deletion in the ORF. Thus, strains NCTC 11168 and 81-176 lack an intact *ars* operon. Interestingly, there is an additional transcriptional regulator upstream of *arsP* in NCTC 11168 and 81-176 (Fig. 1A) and we named this putative regulator *arsR2* based on the fact that its sequence contains an ArsR domain. *arsR2* appears to be transcribed independently from *arsP* and is absent in RM1221. The *ars* genes in strain CB5-28, which is also highly resistant to arsenic (Table 1), were also sequenced in this study (GenBank accession no. EU916833), which revealed that the organization of the *ars* operon in CB5-28 was identical to that of RM1221 (Fig. 1A). There was only one amino acid substitution in ArsP encoded by the entire *ars* operon of CB5-28 compared with the ArsP sequence of RM1221. The immediate flanking gene loci of the *ars* locus are conserved among different *C. jejuni* strains and transcribed divergently from the *ars* locus (Fig. 1A), with a hypothetical ORF located upstream and a *merR* homolog located downstream. The differences in the *ars* locus and the level of resistance to arsenic compounds (Table 1) among RM1221, CB5-28, NCTC 11168, and 81-176 suggested that the *ars* operon contributed to arsenic resistance in *C. jejuni*. This possibility was further examined by the following experiments.

The presence of *arsC* and *acr3* is correlated with increased resistance to arsenic compounds in different *C. jejuni* strains. As the first step to examine if the four-gene (*arsP*, *arsR*, *arsC*, and *acr3*) *ars* operon was associated with enhanced resistance to arsenic compounds, PCR was performed using *arsC*- and *acr3*-specific primers to determine the presence of *arsC* and

TABLE 2. MICs of different arsenic compounds against CB5-28 and its isogenic mutants determined by the agar dilution method and the broth microdilution method[a]

Arsenic compound	MIC (μg/ml) determined by:							
	Agar dilution				Broth microdilution			
	CB5-28	LP001	LP002	LP004	CB5-28	LP001	LP002	LP004
Roxarsone	64	64	64	64	256	256	256	256
Arsenite	64	16 (−4)	8 (−8)	512 (+8)	128	32 (−4)	8 (−16)	1,024 (+8)
Arsenate	1,024	32 (−32)	256 (−4)	1,024	4,096	128 (−32)	512 (−8)	4,096

[a] Numbers in parentheses indicate the fold change compared to CB5-28, which is the wild-type strain. LP001, LP002, and LP004 are the *arsC*, *acr3*, and *arsR* mutants, respectively (see Table S1 in the supplemental material for details).

acr3 in various *C. jejuni* isolates derived from different sources. At the same time, the MICs of the arsenic compounds in these isolates were also measured using the agar dilution method. As shown in Table 1, *arsC* and *acr3* were present in 17 of the 27 isolates examined in this study. In every strain examined, the presence or absence of *arsC* and *acr3* was concurrent, suggesting that the two genes have coevolved. The PCR results indicated that the *ars* operon was widely distributed in *C. jejuni* isolates. Statistical analysis indicated that the presence of the *ars* operon is significantly associated with elevated resistance to arsenite ($P < 0.001$) and arsenate ($P < 0.01$), suggesting that the *ars* operon encodes arsenic resistance mechanisms. Interestingly, the MICs of roxarsone are not significantly different between the strains with and without the *ars* operon ($P > 0.05$), implying that the *ars* operon does not contribute significantly to roxarsone resistance in *C. jejuni* when assayed using the in vitro MIC test.

Contribution of the *ars* operon to arsenic resistance in *C. jejuni*. To define the role of the *ars* operon in arsenic resistance in *Campylobacter*, *arsR*, *arsC*, and *acr3* were disrupted by insertional mutagenesis (for *arsC* and *acr3*) or in-frame deletion (for *arsR*). Each mutant construct was confirmed by PCR (Fig. 1B to D), and the in-frame deletion in the *arsR* mutant was also confirmed by sequence analysis. The mutants were compared with the parent strain CB5-28 for susceptibility to arsenic compounds using two methods, agar dilution and broth microdilution.

According to MIC results from the agar dilution method, inactivation of *arsC* resulted in 4-fold and 32-fold reductions in the MICs of arsenite and arsenate, respectively, while mutation of *acr3* led to eightfold and fourfold reductions in the MICs of arsenite and arsenate, respectively (Table 2). In contrast to the MIC changes associated with the mutation of *arsC* and *acr3*, deletion of *arsR* resulted in an eightfold increase in the MIC of arsenite but had no effect on the MIC of arsenate (Table 2). All three mutants showed no changes in the MIC of roxarsone, indicating that they are not directly associated with resistance to this organic arsenic compound under the in vitro culture conditions. The results from the agar dilution method were also confirmed by the broth dilution method (Table 2). Although the broth dilution method yielded higher MICs than the agar dilution method with each compound, the differences between the mutant strains and the wild-type strain were identical or similar to the results obtained with the agar dilution method (Table 2). With both testing methods, inactivation of *arsC* had a greater effect on the MIC of arsenate than on arsenite, while mutation of *acr3* had a higher impact on the MIC of arsenite (Table 2). This is consistent with the predicted functions of ArsC as an arsenate reductase and Acr3 as an efflux transporter for arsenite. It should be pointed out that the insertional mutation in *arsC* caused a polar effect on *acr3*, because RT-PCR showed that the transcript of *acr3* was greatly reduced (approximately 10-fold) in the *arsC* mutant compared with that in the wild-type strain (data not shown). This polar effect might explain why inactivation of *arsC* also caused a reduction of the MIC of arsenite. Neither the *arsC* mutation nor the *acr3* mutation affected the mRNA levels of *arsP* and *arsR* as determined by real-time RT-PCR (data not shown). Together, these results clearly indicate that *arsC* and *acr3* confer arsenic resistance in *C. jejuni* and deletion of *arsR* increased the resistance to arsenite. Furthermore, we transferred the *arsC* mutation to two additional clinical isolates (CB3-6 and F12469) by natural transformation. In each strain, the mutation led to 8-fold and 16-fold reductions in the MICs of arsenite and arsenate, respectively (Table 3), confirming the finding obtained with CB5-28. Together, these results strongly indicate that the *ars* operon is important for arsenic resistance in *C. jejuni*.

Mutation of the *ars* genes did not affect the susceptibility to antibiotics. To examine if the *ars* operon is associated with antibiotic resistance, we tested the susceptibilities of the wild-type strain CB5-28 and its *arsC*, *arsR*, and *acr3* mutants to antibiotics of different classes, including erythromycin, tilmicosin, ciprofloxacin, enrofloxacin, oxytetracycline, ceftiofur, and polymyxin B. No differences were observed between the wild type and mutants in the susceptibilities to these compounds (data not shown), indicating that the *ars* operon does not confer resistance to antibiotics.

ArsR functions as a repressor for the *ars* operon. The sequence features of ArsR (see Fig. S1 in the supplemental material) and the elevated MIC of arsenite in the *arsR* mutant (Table 2) suggested that ArsR functions as a repressor for the

TABLE 3. MICs of arsenic in *C. jejuni* strains CB3-6 and F12469 and their *arsC* mutants as determined by agar dilution

Arsenic	MIC (μg/ml)[a]			
	CB3-6	CB3-6 (*arsC*::Kan^r)	F12469	F12469 (*arsC*::Kan^r)
Roxarson	32	32	128	128
Arsenite	64	8 (−8)	64	8 (−8)
Arsenate	1,024	64 (−16)	2,048	128 (−16)

[a] Numbers in parentheses indicate the fold change compared to the corresponding wild-type strain.

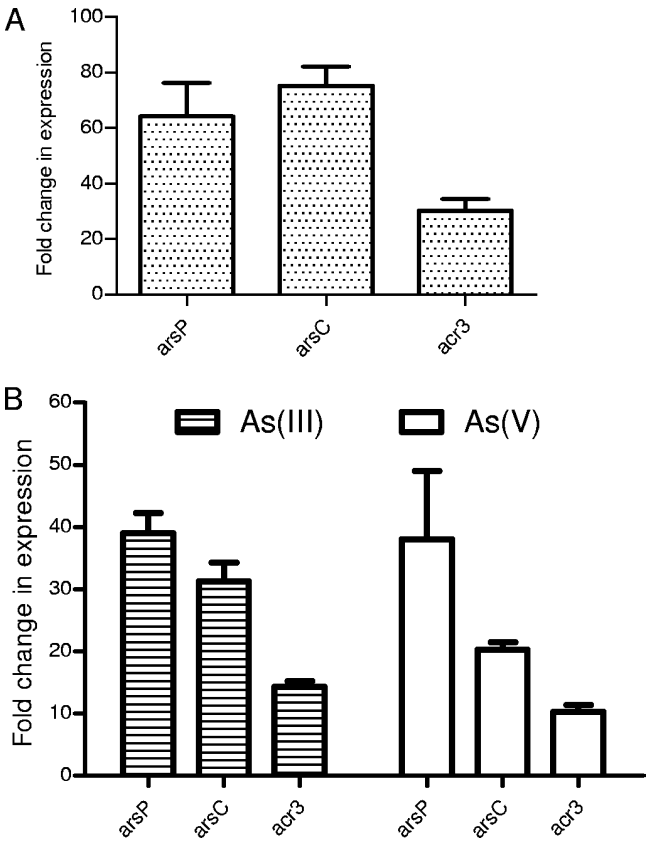

FIG. 2. Expression levels of the *ars* genes as determined by qRT-PCR. (A) Elevated expression of *arsP*, *arsC*, and *acr3* in the *arsR* deletion mutant (LP004) compared to the wild-type CB5-28. (B) Induction of *arsP*, *arsC*, and *acr3* in CB5-28 by arsenite [As(III)] and arsenate [As(V)] at a subinhibitory concentration. In both panels, each bar represents the mean ± standard deviation of three independent experiments.

ars operon. To test this hypothesis, we determined the expression of the *ars* genes in the Δ*arsR* mutant. Deletion of *arsR* did not produce a polar effect on the downstream genes, as confirmed by RT-PCR (data not shown). In fact, qRT-PCR showed that deletion of *arsR* increased expression of the other three genes in the *ars* operon. Compared with the wild-type strain, *arsP*, *arsC*, and *acr3* in the *arsR* deletion mutant showed 64-, 75-, and 30-fold increases in transcription, respectively (Fig. 2A). This result further indicates that ArsR functions as a repressor inhibiting the expression of the *ars* operon in *Campylobacter*.

The *ars* operon is inducible by arsenite and arsenate. To determine if the expression of the *ars* operon is inducible by arsenic compounds, strain CB5-28 was cultured in MH broth with arsenite and arsenate. The transcription levels of *arsP*, *arsC*, and *acr3* in these cultures were compared with those grown in MH broth without inducers using qRT-PCR. As shown in Fig. 2B, the expression of *arsP*, *arsC*, and *acr3* was induced 39-, 31-, and 14-fold, respectively, by arsenite, and 38-, 20-, and 13-fold, respectively, by arsenate. This result clearly indicated that the *ars* operon in *Campylobacter* is strongly induced by arsenic compounds, which suggests that this system is subject to transcriptional regulation. Interestingly, the induction of the *ars* genes was not at the same level, with *arsP* (close to the *ars* promoter) showing the highest and *acr3* (distant from the *ars* promoter) the lowest fold changes in expression (Fig. 2B). This difference is probably due to the polar expression effect, as observed with the *ars* operons in other bacteria (22, 29).

Identification of the promoter for the *ars* operon. To understand the regulatory mechanism of the *ars* operon, we first identified its transcription start using primer extension. The electropherogram showed a single distinct peak corresponding to a cDNA fragment of 214 bases, which mapped the transcription start to 20 nucleotides upstream of the start codon of *arsP* (Fig. 3A and C). Based on the result of primer extension and the reported consensus sequences of *Campylobacter rpoD* promoters (37), the predicted −10 and −35 sequences were also identified (Fig. 3A). Overlapping with the −35 site, there is an 18-bp inverted repeat forming a dyad structure, suggesting a potential binding site for a regulatory protein. Interestingly, similar inverted repeats are also present in the promoters of the *ars* operons of *Bacillus subtilis* and *Synechocystis* species (29, 45), and the core sequence [ATCAA$(N)_6$TTGAT] is conserved among the three organisms (Fig. 3B). In *Synechocystis* species, the inverted repeat was mapped in the binding site of ArsR (29).

ArsR binds specifically to the promoter of the *ars* operon. The presence of an HTH motif in ArsR (see Fig. S1 in the supplemental material) suggests that it is a DNA-binding protein. In addition, an 18-bp inverted repeat is present in the promoter region of *ars* (Fig. 3A), suggesting a potential binding site for ArsR. To determine whether ArsR directly interacts with the *ars* promoter, rArsR protein was expressed in *Escherichia coli*. The rArsR was shown as a 13-kDa protein on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (data not shown), comparable with the molecular mass (12.2 kDa) calculated from the deduced amino acid sequence of ArsR. As shown with EMSA, rArsR formed complexes with the 164-bp DNA fragment containing the promoter of the *ars* operon in a dose-dependent manner (Fig. 3D) but did not bind to the control DNA fragment amplified from the coding region of *arsP* (data not shown). To further confirm the binding specificity of ArsR and determine if the inverted repeat in the *ars* promoter region was involved in the binding, we performed competition experiments using EMSA. A 50-bp DNA fragment containing the inverted repeat was synthesized using complementary oligonucleotides CompF and CompR (see Table S2 in the supplemental material). In the competition reaction, this unlabeled DNA fragment inhibited the binding of ArsR to the *ars* promoter DNA (Fig. 3E). On the contrary, the control DNA (coding region of *arsP*) did not block the binding of ArsR to the promoter even at a 200-fold molar excess of the promoter DNA (Fig. 3F). ArsR also did not show binding to an unrelated promoter of *cmeABC* (data not shown) controlled by CmeR, a TetR family transcription regulator (23). Together, these results indicate that ArsR binds directly to the promoter of the *ars* operon, specifically to the region containing the inverted repeat.

Binding of ArsR to the *ars* promoter is inhibited by arsenite. Since arsenic compounds induce the expression of the *ars* operon (Fig. 2) and there is a conserved metal binding motif (ELCVCDL) (48) in the ArsR (see Fig. S1 in the supplemental

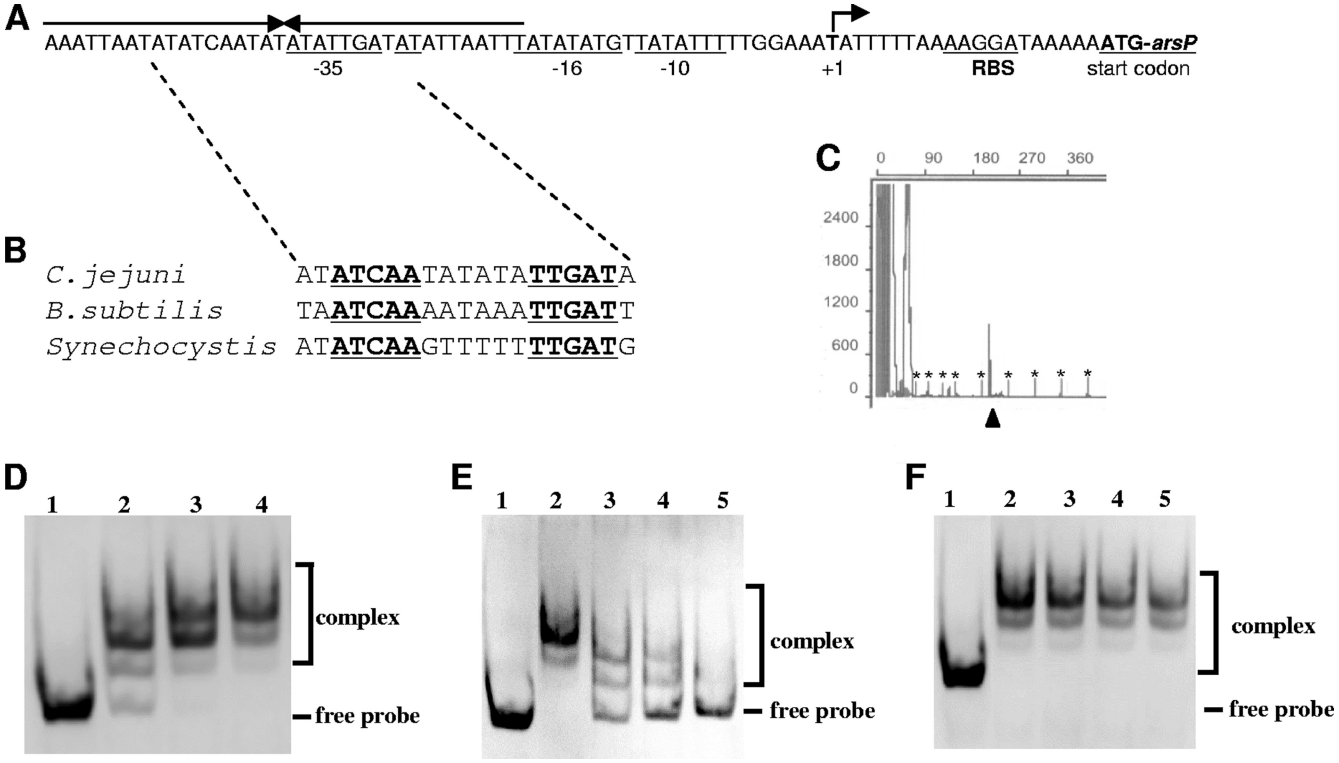

FIG. 3. Regulation of the *ars* operon. (A) Sequence features of the *ars* promoter. The start codon (ATG) of *arsP* is bold. The transcript start (base T) is marked as +1, and a curved arrow indicates the direction of transcription. The predicated ribosomal binding site (RBS) is underlined. The putative −10, −16, and −35 boxes are underlined. The inverted repeat is overlined with inverted arrows. (B) Alignment of the ArsR binding sites among *C. jejuni*, *B. subtilis*, and *Synechocystis*. The conserved sequences are bold and underlined. (C) An electropherogram showing the result of primer extension analysis of the *ars* promoter. The extension product is indicated by an arrowhead. The 5-6-carboxyfluorescein-labeled size standards are marked with asterisks. (D) Binding of ArsR to the *ars* promoter as determined by EMSA. A 164-bp DIG-labeled DNA fragment containing the *ars* promoter was incubated with 0 ng (lane 1), 5 ng (lane 2), 10 ng (lane 3), and 20 ng (lane 4) of rArsR. (E) Competition assay to confirm the binding specificity of ArsR. Each binding reaction was conducted with the DIG-labeled promoter DNA and 20 ng of ArsR in the presence of the competing DNA (a 50-bp probe containing the IR). The competing DNA was added at a zerofold (lane 2), 50-fold (lane 3), 100-fold (lane 4), and 200-fold (lane 5) molar excess of the DIG-labeled promoter DNA. Lane 1 contains the DIG-labeled promoter DNA only (no rArsR). (F) Competition assay using a DNA fragment amplified from the coding sequence of *arsP*, which was added with no (lane 2), 50-fold (lane 3), 100-fold (lane 4), and 200-fold (lane 5) excess of the DIG-labeled promoter DNA. Lane 1 contains the DIG-labeled promoter DNA only.

material), we suspected that arsenic compounds induce *ars* expression by inhibiting the interaction of ArsR with the target promoter. To test this hypothesis, we investigated the effect of arsenite and arsenate on the binding of ArsR to the promoter DNA in EMSA. The results demonstrated that arsenite inhibited the formation of the ArsR-DNA complex in a dose-dependent manner and completely released ArsR from the DNA at a concentration of 1 mM (Fig. 4). On the other hand, arsenate did not inhibit the binding of ArsR to the promoter DNA even at a concentration of 32 mM. These

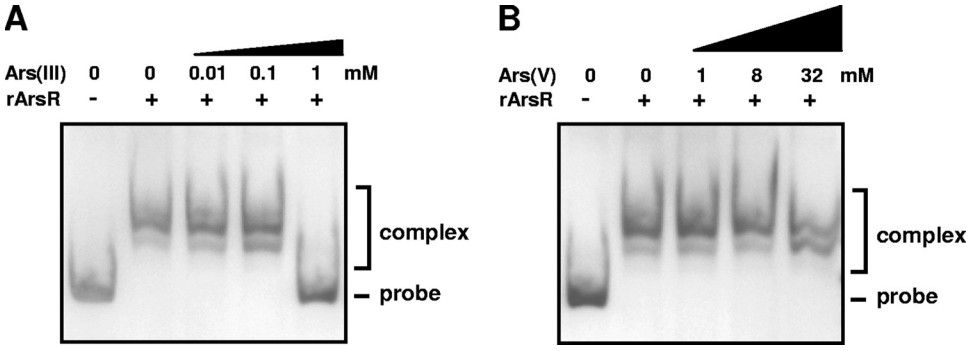

FIG. 4. Effect of arsenite (A) and arsenate (B) on the formation of ArsR-DNA complexes as determined by EMSA. The concentrations of arsenic used in the reaction are indicated at top of each panel. Each reaction was performed with 5 ng of DIG-labeled *ars* promoter DNA. +, 20 ng of rArsR was added in the reaction; −, rArsR was absent in the reaction.

results suggest that arsenite directly inhibits the interaction of ArsR with the target DNA, while arsenate is inefficient in the inhibition.

DISCUSSION

In this study, a four-gene *ars* operon was identified that contributes to arsenic resistance in *C. jejuni*. Genes in this operon encode two putative membrane transporters (ArsP and Acr3), a transcriptional regulator (ArsR), and an arsenate reductase (ArsC). Compared to the *ars* operons in other bacteria, the *C. jejuni ars* is atypical in terms of gene contents because it encodes two membrane transporters that show little sequence homology with the ArsB described in *E. coli* and other bacteria. There are two unrelated families of arsenite transporters in bacteria: the ArsB family and the Acr3 family (40, 41). The ArsB family has so far been identified only in bacteria, while the Acr3 family is present in bacteria, fungi, and archaea. ArsB has 12 membrane-spanning segments and extrudes both arsenite and antimonite (40). In contrast, Acr3 has 10 predicted transmembrane helices and is an arsenite-specific transporter in bacteria. Limited sequence homology exists between ArsB and Acr3. ArsB either functions alone using membrane potential in the efflux of arsenite or partners with ArsA (ATPase) to form a highly efficient ATP-driven arsenite efflux pump. Acr3 utilizes membrane potential for the efflux of arsenite. The majority of bacterial *ars* operons possess *arsB* instead of *acr3* (41), which has been reported only in a few bacteria (29, 45, 51). However, a recent survey of soil bacteria using *acr3*-specific PCR analysis indicated that the *acr3* gene is widely distributed in different bacterial species (1). Results from this study indicate that the *acr3* gene is frequently present in various *C. jejuni* strains isolated from different sources (Table 1). In addition, an *acr3* homolog is also present in two recently sequenced *C. jejuni* strains, including 81116 and a clinical isolate from Thailand (36, 38). Together, these findings suggest that the *acr3* class of arsenic transporters is more commonly present in bacteria than previously expected.

In the *ars* operon described in this study (Fig. 1A), the first ORF (*arsP*) encodes a putative permease of the major facilitator family. Predicted with TMHMM 2.0, ArsP has eight predicted transmembrane helices and a large hydrophilic loop (67 amino acids) in the central portion (between helices four and five) of the protein. The central hydrophilic loop is predicted to be outside the membrane. ArsP has little sequence homology with proteins encoded by either *arsB* or *acr3*. Since *arsP* is in the *ars* operon and is controlled by ArsR (Fig. 2), it is possible that ArsP also contributes to arsenic resistance in *Campylobacter*. However, attempts to inactivate the *arsP* gene by in-frame deletion were not successful for unknown reasons. Thus, how ArsP contributes to arsenic resistance in *Campylobacter* remains to be determined in future studies.

Analysis of multiple clinical isolates revealed a statistically significant association between harboring this *ars* operon and elevated resistance to arsenite and arsenate (Table 1). Furthermore, inactivation of *arsC* and *acr3* in *Campylobacter* decreased its resistance to arsenite and arsenate (Tables 2 and 3). These findings indicate that this *ars* operon is an important player in conferring arsenic resistance in *Campylobacter*. Surprisingly, several strains (CT3-7, CT1-20, CT4-3, and H52022) appeared to harbor this operon based on the PCR result but did not show substantially elevated resistance to arsenic compared to those strains that lacked the operon (Table 1). A possible explanation for this discrepancy is that the *ars* operon in these strains is not functional due to sequence degeneration. This possibility remains to be verified by sequencing the *ars* operon in the strains, which will determine if frameshift mutations or other changes in the *ars* genes have occurred in the strains. Interestingly, inactivation of *arsC* and *acr3* did not affect the susceptibility to roxarsone (an organic arsenic compound), suggesting that this operon is not directly involved in the detoxification of roxarsone. However, roxarsone is eventually converted to inorganic arsenic in poultry litter (8, 49). Thus, this *ars* system would play an indirect role in the resistance to roxarsone. In addition to the *ars* operon, *C. jejuni* also has other putative arsenic resistance genes. For example, the updated annotation of strain NCTC 11168 (http://www.lshtm.ac.uk/pmbu/crf/Cj_updated.art) indicates that this strain has three additional *ars* genes, including CJ0258 (an *arsR* homolog), CJ0717 (an *arsC* homolog), and CJ1187c (an *arsB* homolog). These individual genes are located far apart on the chromosome and have not been functionally characterized. Whether they contribute to arsenic resistance in *Campylobacter* awaits further investigation.

It was shown in this study that ArsR encoded by the second gene in the operon serves as a repressor for the *ars* system in *C. jejuni*. This conclusion is based on the fact that deletion of *arsR* greatly increased the expression of the *ars* genes (Fig. 2A) and the resistance to arsenite (Table 2). Interestingly, the *arsR* deletion mutant (LP004) did not affect the MIC of arsenate (Table 2), suggesting that the elevated expression of the *ars* operon in LP004 did not make a difference in the resistance to arsenate. This observation may be explained by the possibility that the inducible expression of the *ars* operon in the presence of arsenate is as effective as the *arsR* deletion in conferring the resistance to arsenate. Alternatively, multiple mechanisms are involved in the detoxification of arsenate in *Campylobacter* and overexpression of the *ars* system alone does not necessarily show an obvious change in the resistance to arsenate.

As a transcriptional repressor, ArsR binds specifically to the promoter region of the *ars* operon (Fig. 3D). Since the inverted repeat sequence inhibited the binding of ArsR to the promoter DNA (Fig. 3E), the dyad symmetry in the promoter region is likely to be the binding site of ArsR. The ArsR binding site overlaps with the −35 site of the *ars* promoter (Fig. 3A). Thus, binding by ArsR would presumably block the interaction of RNA polymerases with the promoter and inhibit the initiation of transcription. Interestingly, the inverted repeat sequence is also partially present in the *ars* promoters of *B. subtilis* and *Synechocystis* spp. (29, 45), and the core dyad sequence ATC $AA(N)_6$TTGAT was conserved among the three organisms (Fig. 3B). In *E. coli*, $TCAT(N)_7$TTTG was found to be the consensus binding site for the ArsR of both plasmid and chromosomal origins (58), which is also somewhat similar to the conserved ArsR binding site identified in this study. These observations suggest that ArsR proteins from bacterial species have conserved certain binding specificity. As a member of the metalloregulatory protein family, the ArsR in *Campylobacter* is expected to bind to DNA as homodimers. As shown by EMSA (Fig. 3D), up to three shifted bands were observed with the

promoter DNA upon the addition of ArsR, which might represent different forms of the DNA-protein complexes. This possibility remains to be examined in future studies. With the SmtB metalloregulatory protein in cyanobacteria, it has been known that up to four dimers can bind to a single inverted repeat (11, 21, 28).

The expression of the *ars* operon in *Campylobacter* was inducible by both arsenite and arsenate (Fig. 2B), but only arsenite inhibited the binding of ArsR to the operator DNA (Fig. 4), indicating that arsenite is the true inducer of ArsR. Similar findings were observed with arsenic induction in *E. coli* (56). The discrepancy between the in vivo induction and the in vitro binding inhibition can be explained by the fact that arsenate is converted to arsenite by ArsC in bacterial cells. It was possible that the added arsenate in the *Campylobacter* cultures was reduced to arsenite by ArsC, which then inhibited the function of ArsR, leading to induction of the *ars* operon (Fig. 2B). In *E. coli*, it has been shown that arsenite binds to the Cys residues in the metal binding box of ArsR, and this interaction presumably causes a conformational change in ArsR, leading to the dissociation of ArsR from the promoter DNA (47). The ArsR in *C. jejuni* also has the highly conserved metal binding motif (see Fig. S1 in the supplemental material). Thus, it is conceivable that induction of ArsR by arsenite in *Campylobacter* also occurs at this site, which will be confirmed in future studies. Notably, the rArsR is unstable during purification and storage. To avoid this problem, we adopted a rapid purification protocol and froze the purified rArsR in single-use aliquots immediately after purification. Each aliquot was used only once for EMSA. The instability of the purified rArsR is probably due to the formation of intra- and intermolecular disulfide bonds resulting from the oxidation of the free thiol groups of the cysteine residues. This problem was observed with other purified regulatory proteins, such as QacR and MerR (14, 46). The *Campylobacte*r ArsR has three Cys residues, two in the metal binding box and one at the C terminus (see Fig. S1 in the supplemental material). Their involvement in rapid formation of disulfide bonds may be responsible for the loss of binding activity of rArsR during storage.

As a major food-borne human pathogen, *Campylobacter* spp. are commonly present in farm animals and their production environment (18). Particularly, poultry harbors large numbers of *Campylobacter* bacteria and are considered a natural habitat for this organism (61). Since arsenic compounds are frequently used for growth promotion and disease prevention in poultry production (6), *Campylobacter* bacteria are likely exposed to arsenic at a level that is higher than that in the natural environment. Work presented in this study identifies a new arsenic-sensing and resistance mechanism in *C. jejuni*, which may have evolved to facilitate *Campylobacter* adaptation in the poultry production system. Our findings provide new insights into the adaptive mechanisms of *Campylobacter* bacteria in animal reservoirs and open a new direction for us to understand how antimicrobial usage in animal production affects the physiology and ecology of food-borne pathogens.

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